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L1: Entry 8 of 10 File: USPT Jul 3, 2001

US-PAT-NO: 6255081
DOCUMENT-IDENTIFIER: US 6255081 B1

TITLE: Thermostable flap endonuclease derived from hyperthermophile bacterium belonging to the genus pyrococcus

DATE-ISSUED: July 3, 2001

INVENTOR-INFORMATION:

NAME	CITY	STATE	ZIP CODE	COUNTRY
Matsui; Ikuo	Ibaraki			JP
Ishikawa; Kazuhiko	Ibaraki			JP
Kosugi; Yoshitsugu	Ibaraki			JP
Matsui; Eriko	Ibaraki			JP
Kawasaki; Satoko	Chiba			JP

ASSIGNEE-INFORMATION:

NAME	CITY	STATE	ZIP CODE	COUNTRY	TYPE CODE
Director - General of Agency of Industrial Science and Technology	Tokyo			JP	03

APPL-NO: 09/ 175973 [PALM]
DATE FILED: October 21, 1998

PARENT-CASE:

This is a divisional application based on U.S. application Ser. No. 09/146,319 filed Sep. 3, 1998, the contents of which are incorporated by reference.

INT-CL: [07] C12 P 19/34, C12 N 9/22, C12 N 15/55

US-CL-ISSUED: 435/91.1; 435/199, 435/252.3, 435/252.33, 435/320.1, 536/23.2
US-CL-CURRENT: 435/91.1; 435/199, 435/252.3, 435/252.33, 435/320.1, 536/23.2

FIELD-OF-SEARCH: 435/320.1, 435/194, 435/252.3, 435/252.33, 435/91.1, 536/23.2

PRIOR-ART-DISCLOSED:

U.S. PATENT DOCUMENTS

Search Selected Search ALL Clear

	PAT-NO	ISSUE-DATE	PATENTEE-NAME	US-CL
☐	5843669	December 1998	Kaiser et al.	435/6

☐	5846717	December 1998	Brow et al.	435/6
☐	5874283	February 1999	Harrington et al.	435/252.3

FOREIGN PATENT DOCUMENTS

FOREIGN-PAT-NO	PUBN-DATE	COUNTRY	US-CL
9-239440	April 1997	JP	

ART-UNIT: 162

PRIMARY-EXAMINER: Patterson, Jr.; Charles L.

ATTY-AGENT-FIRM: Foley & Lardner

ABSTRACT:

The present invention relates to a thermostable Flap endonuclease whose optimum temperature is 75.degree. C. or more and DNA coding for (a) a protein consisting of the amino acid sequence shown in SEQ ID NO:2 or (b) a protein with Flap endonuclease activity, consisting of an amino acid sequence where in the amino acid sequence (a), one or more amino acids are deleted, substituted or added. According to the present invention, there is provided a thermostable Flap endonuclease whose optimum temperature for reaction is 75.degree. C. or more. Further, this enzyme is thermally stable, so it becomes possible to develop new techniques of conducting artificial homologous recombination or genetic shuffling highly efficiently by coupling the enzyme reaction with PCR.

8 Claims, 2 Drawing figures
Exemplary Claim Number: 1
Number of Drawing Sheets: 2

BRIEF SUMMARY:

1 BACKGROUND OF THE INVENTION

2 1. Field of the Invention

3 The present invention relates to a thermostable Flap endonuclease effective for genetic recombination and genetic shuffling based on low homology, as well as to a gene thereof.

4 2. Description of the Prior Art

5 There are a method for random mutation of a broad region in a gene and a method for random mutation of a local region in a gene. In the former method, polymerase chain reaction (PCR) is applied such that a specific nucleotide is deleted to induce mutation at the time of replication of the target gene, and in the latter method, PCR using mixed primers is applied to mutate the target site. However, there is still no method for highly efficient induction of genetic recombination or genetic shuffling based on low homology in vitro. A speculative mechanism of genetic recombination and genetic shuffling in vivo

is shown in FIG. 1. Step 1 of FIG. 1 shows formation of a single-stranded overhang by a 3'-5' exonuclease; step 2 shows formation of temporary nucleotide base pairs based on low homology; step 3 shows repair of the gap by DNA polymerase and formation of a Flap structure; step 4 shows removal of the Flap single strand by a Flap endonuclease; and step 5 shows ligation of nicks by a DNA ligase. However, the properties of the enzymes catalyzing the respective steps are not fully elucidated. The Flap endonuclease is an enzyme catalyzing step 4. As shown in FIG. 2, the Flap endonuclease specifically recognizes the Flap structure in DNA and cleaves the single strand (called Flap), and this enzyme is found in mammalian cells and yeast cells. Since its origin is organisms living at normal temperatures, this enzyme has poor thermostability and thus is not suitable for artificial genetic shuffling reaction, including PCR.

6 Because the conventional Flap endonuclease is unstable at high temperatures, it cannot be used to develop methods where genetic recombination or genetic shuffling based on low homology in vitro is induced at high temperatures. However, if a thermostable Flap endonuclease functioning stably at high temperatures can be obtained, it becomes possible to develop new techniques of conducting artificial homologous recombination or genetic shuffling highly efficiently by coupling the enzyme reaction with PCR. Accordingly, development of the thermostable Flap endonuclease stable at high temperature has long been desired.

7 SUMMARY OF THE INVENTION

8 Under these circumstances, the object of the present invention is to provide a novel thermostable Flap endonuclease and a gene thereof.

9 To achieve this object, the present inventors directed their attention to a hyperthermophile bacterium growing at 90 to 100.degree. C., and as a result, a gene presumed to bring about the activity of the present enzyme was found in a gene sequence from the bacterium. Further, the enzyme was produced in E. coli by use of said gene, and it was confirmed that this enzyme is stable at high temperatures (75.degree. C. or more) and exhibits structurally specific endonuclease activity. The present invention was thereby completed.

10 That is, the present invention relates to a thermostable Flap endonuclease whose optimum temperature is 75.degree. C. or more.

11 Further, the present invention relates to a DNA coding for the following protein (a) or (b): (a) a protein consisting of the amino acid sequence shown in SEQ ID NO:2; and (b) a protein with Flap endonuclease activity, consisting of an amino acid sequence where in the amino acid sequence (a), one or more amino acids are deleted, substituted or added.

12 The DNA is a DNA specifically shown in SEQ ID NO:1.

13 The addition, deletion or substitution of amino acids can be effected using site-directed mutagenesis known in the art (see e.g. Nucleic Acid Research, Vol. 10, No. 20, pp. 6487-6500 (1982)). The number of one or more amino acids added, deleted or substituted is the number of amino acids which can be added, deleted or substituted by site-directed mutagenesis.

14 Further, the present invention relates to a recombinant vector comprising said DNA.

15 Further, the present invention relates to a transformant transformed with the recombinant vector comprising said DNA.

16 Further, the present invention relates to a process for producing a thermostable Flap endonuclease, which comprises culturing said transformant in a medium to produce the thermostable Flap endonuclease.

DRAWING DESCRIPTION:

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 shows a mechanism of homogeneous recombination and genetic shuffling in vivo in which the thermostable Flap endonuclease is involved. The Flap endonuclease catalyzes step 4.

FIG. 2 shows one example of the DNA structure (SEQ ID NOS 5, 6, & 7, respectively) which can serve as a substrate for the Flap endonuclease. The single-stranded portion is called a Flap. The arrow indicates the site to be cleaved by this enzyme.

DETAILED DESCRIPTION:

1 DETAILED DESCRIPTION OF THE INVENTION

2 Hereinafter, the present invention is described in detail.

3 The hyperthermophilic bacterium used in the present invention is sulfur-metabolizing thermophilic archaebacterium, Pyrococcus horikoshii JCM9974.

4 The thermostable Flap endonuclease of the present invention was obtained in the following manner. PCR was applied using chromosomal DNA as a template from Pyrococcus horikoshii JCM9974 and the upper and lower primers shown in Example 6, and a DNA fragment containing a Flap endonuclease gene was isolated.

5 This gene was inserted into protein expression plasmid pET 15b, then the resulting recombinant plasmid was integrated in E. coli, and the transformed E. coli was cultured in a medium to produce the present enzyme. The produced thermostable Flap endonuclease was isolated and purified by heat treatment and column chromatography. It was confirmed that the purified Flap endonuclease is a protein with a molecular weight of about 40,000 and is an enzyme which recognizes the Flap type DNA substrate shown in FIG. 2 and hydrolyzes a single-stranded portion (Flap portion) in an oligonucleotide.

6 The activity of the present enzyme did not decrease even after treatment of the enzyme at 95.degree. C. for several hours in 50 mM phosphate buffer, pH 7.5 containing 1 M NaCl. Further, the optimum pH for activity was 6.0 to 8.0, and the optimum temperature was about 75.degree. C. or more at pH 8.

7 EFFECT OF THE INVENTION

8 According to the present invention, there is provided a thermostable Flap endonuclease whose optimum temperature for reaction is 75.degree. C. or more. Further, the present enzyme is thermally stable, so it becomes possible to develop new techniques of conducting artificial homologous recombination and genetic shuffling highly efficiently by coupling the enzyme reaction with PCR.

9 EXAMPLES

10 Hereinafter, the present invention is described in detail with reference to Examples, which, however, are not intended to limit the technical scope of the present invention.

11 Example 1

12 Culture of the Microorganism

13 Pyrococcus horikoshii JCM9974 was cultured in the following manner.

14 Dissolved in 1 L were 13.5 g sodium chloride, 4 g Na.sub.2 SO.sub.4, 0.7 g KCl, 0.2 g NaHCO.sub.3, 0.1 g KBr, 30 mg H.sub.3 BO.sub.3, 10 g MgCl.sub.2. 6H.sub.2 O, 1.5 g CaCl.sub.2, 25 mg SrCl.sub.2, 1.0 ml resazurin solution (0.2 g/L), 1.0 g yeast extract and 5 g Bacto-trypton, and this solution was adjusted to pH 6.8 and sterilized under pressure. Then, dry-sterilized elementary sulfur was added thereto at a concentration of 0.2%, and this medium was rendered anaerobic by saturation with argon, and Pyrococcus horikoshii JCM9974 was inoculated thereinto. Whether the medium became anaerobic or not was confirmed by failure of Na.sub.2 S to cause the resazurin to turn pink in the culture solution upon addition of a Na.sub.2 S solution. This culture solution was incubated at 95.degree. C. for 2 to 4 days, and the resulting culture was harvested by centrifugation at 5000 rpm for 10 minutes to give 1 g of the microorganism.

15 Example 2

16 Preparation of Chromosomal DNA

17 Chromosomal DNA from Pyrococcus horikoshii JCM9974 was prepared in the following manner. The microorganism obtained (0.1 g) in Example 1 was washed twice with 10 mM Tris buffer (pH 7.5), 1 mM EDTA and enclosed in an InCert Agarose (FMC Co., Ltd.) block. This block was treated with 1% N-lauroylsarcosine and 1 mg/ml protease K, whereby the chromosomal DNA was separated and prepared in the Agarose block.

18 Example 3

19 Preparation of Library Clones Containing the Chromosomal DNA

20 The chromosomal DNA obtained in Example 2 was partially digested with restriction enzyme HindIII, and then a fragment of about 40-kb in length was prepared by agarose gel electrophoresis. This DNA fragment was ligated by T4 ligase to Bac vector pBAC108L (Ung-Jon Kim et al., Nucleic Acid Research, 20 (5), 1083-1085 (1992)) and pFOSI (Ung-Jon Kim et al., Nucleic Acid Research, 20(5), 1083-1085 (1992)), both vectors having been completely digested with restriction enzyme HindII. If the former vector was used, the DNA after ligation was immediately introduced by electroporation into E. coli. If the latter vector pFOS1 was used, the DNA after ligation was packaged into .lambda.-phage particles in vitro by GIGA Pack Gold (Stratagene) and these particles were infected into E. coli whereby the DNA was introduced into the E. coli. The resulting E. coli populations resistant to antibiotic chloramphenicol were used as BAC and Fosmid libraries respectively. From the libraries, clones suitable for covering the chromosome of Pyrococcus horikoshii JCM9974 were selected and alignment of the clones was conducted.

21 Example 4

22 Nucleotide Sequencing of each of the BAC and Fosmid Clones

23 Nucleotide sequencing of each of the aligned BAC and Fosmid clones was conducted in the following manner. The BAC or Fosmid clone DNA recovered from the E. coli was fragmented by ultrasonication, and 1- and 2-kb long DNA fragments were recovered by agarose gel electrophoresis. Shotgun clones with the fragments inserted into a HincII restriction enzyme site on plasmid vector pUC118, that is, 500 clones derived from each of the BAC and Fosmid clones, were prepared. The nucleotide sequence of each shotgun clone was determined by Automatic Nucleotide Sequence Reader 373 or 377 manufactured by Perkin Elmer, ABI. The nucleotide sequences obtained from the respective shotgun clones were linked and compiled by nucleotide sequence automatic linking software Sequencer to determine the whole nucleotide sequence of each of the BAC and Fosmid clones.

24 Example 5

25 Identification of the Flap Endonuclease Gene

26 The nucleotide sequence of each of the BAC and Fosmid clones determined above was analyzed by a large computer to identify a gene coding for the Flap endonuclease. The sequence of this gene is shown in SEQ ID NO:1. The amino acid sequence deduced from this nucleotide sequence is shown in SEQ ID NO:2.

27 Example 6

28 Construction of the Expression Plasmid

29 For the purpose of creating restriction enzyme sites (NdeI and XhoI) upstream and downstream from the structural gene, DNA primers were synthesized, and by PCR, the restriction enzyme sites were created in regions upstream and downstream from the gene.

30 Upper primer (SEQ ID NO:3): 5'-GGGAATTCCTGCAGATCGCATATGGGTGTTCCTATCGGTGAC-3'

31 Lower primer (SEQ ID NO:4): 5'-ACTAATCCCGGGTACCTCGAGGCTATAGACTTTAGGGTTTCT-3'

32 After PCR, the product was completely digested (37.degree. C., 2 hours) with the restriction enzymes (NdeI and XhoI) and the structural gene was then purified. pET-15b (Novagen) was cleaved with restriction enzymes NdeI and XhoI, then purified and ligated by T4 ligase to the above structural gene at 16.degree. C. for 2 hours. A part of the ligated DNA was introduced into E. coli-XL 1-Blue MRF1 competent cells to give a transformant colony. The expression plasmid was purified from the resulting colony by the alkali method (Sambrook, J. et al., Molecular Cloning: A laboratory Manual, 2nd edn., Cold Spring Harbor Laboratory (1989)).

33 Example 7

34 Expression of the Recombinant Gene

35 E. coli competent cells (E. coli BL21 (DE3) available from Novagen) were fused and 0.1 ml cells were transferred to a Falcon tube. The expression plasmid

solution (0.005 mL) was added to the cells, left on ice for 30 minutes, and heat-shocked at 42.degree. C. for 30 seconds, and 0.9 mL SOC medium was added thereto, and the cells were cultured at 37.degree. C. for 1 hour under shaking. Then, the cells were plated on a 2YT agar plate containing ampicillin and cultured at 37.degree. C. overnight to give a transformant. This transformant has been deposited as FERM P-16389 on Aug. 18, 1997 with the National Institute of Bioscience and Human-Technology, Agency of Industrial Science and Technology, Japan.

36 This transformant was cultured in ampicillin-containing 2YT medium, 2L, until its absorbance at 600 nm reached 1, and then IPTG (isopropyl-.beta.-D-thiogalactopyranoside) was added thereto, followed by culturing for further 6 hours. After culturing, the transformant was harvested by centrifugation (6,000 rpm, 20 minutes) to give 30 g of the microorganism.

37 Example 8

38 Purification of the Thermostable Enzyme

39 The harvested microorganism (30 g) was frozen at -20.degree. C. and thawed, then alumina in an amount as twice as that of the microorganism and 1 mg DNase were added thereto, and the microorganism was disrupted, followed by adding a 5-fold excess amount of 10 mM Tris-HCl buffer (pH 8.0) to give a suspension. The resulting suspension was heated at 85.degree. C. for 30 minutes and centrifuged (11,000 rpm, 20 minutes), and the supernatant was subjected to affinity chromatography on a Ni column (using Novagen, His-Bind metal chelation resin & His-Bind buffer kit). A fraction eluted with 60 mM imidazole was adsorbed onto a HiTrap SP (Pharmacia) and eluted with a concentration gradient of NaCl to give an active fraction. The resulting active fraction solution was further applied to a Hitrap Heparin column (Pharmacia) and eluted with a concentration gradient of NaCl to give 1 mg purified enzyme.

40 Example 9

41 Conditions for the Enzyme Reaction

42 (1) Synthetic Oligonucleotides

43 All oligonucleotides were synthesized by Grainer Japan Co., Ltd. The names and sequences of the respective oligonucleotides are as follows.

44 F.sub.br strand (SEQ ID NO:5): 5'-GGACTCTGCCTCAAGACGGTAGTCAACGTG-5'

45 F.sub.adj strand (SEQ ID NO:6): 5'-CTGCCATCAGTTGCAC-3'

46 Flap strand (SEQ ID NO:7): 5'-CCTGAGACGGAGTTTCAATCCTGACGAACTGTAG-5'

47 (2) Preparation of the Flap Substrate (FIG. 2)

48 The Flap strand was labeled at the 5'-terminal thereof with .sup.32 P by means of [.gamma.-.sup.32 P]ATP and with T.sub.4 polynucleotide kinase. The labeled Flap strand was boiled together with the F.sub.br and F.sub.adj strands in 20 mM Tris-HCl buffer, pH 7.4, 150 mM NaCl and then annealed by gradually lowering the temperature of the solution to 4.degree. C.

49 (3) Flap endonuclease Activity

50 The .sup.32 P-labeled Flap substrate and the enzyme (400 fmol) were added to 15 ml of 50 mM Tris-HCl buffer (pH 8, 1.5 mM MgCl.sub.2, 0.5 mM .beta.-mercaptoethanol, 100 mg/ml bovine serum albumin) and reacted at 50 .degree. C. for 30 minutes. Then, 15 ml of 95% formamide, 10 mM EDTA and 1 mg/ml xylene cyanol were added to stop the enzyme reaction. Further, this reaction solution was heated at 95.degree. C. for 5 minutes and electrophoresed at 2000 V for 2 hours in 15% polyacrylamide gel (16.times.45 cm) containing 7 M urea and 1.times.TBE (89 mM Tris, 89 mM boric acid, 2 mM EDTA, pH 8). The reaction product was imaged and quantified by Molecular Imager GS-525 (Bio-Rad). One unit activity is defined as the amount of the enzyme causing 1 fmol Flap substrate to be decomposed under usual conditions for measuring Flap endonuclease activity. Properties of the enzyme

51 (1) Substrate Specificity

52 The present enzyme does not act on double-stranded DNA or single-stranded DNA and has the endonuclease activity of cleaving off the single-stranded DNA (Flap) from the DNA structure shown in FIG. 2. In the substrate shown in FIG. 2, the arrow indicates the cleavage site from which the 20-mer single-stranded DNA is released. Further, the activity of this enzyme does not depend on the nucleotide sequence of DNA.

53 (2) Optimum pH

54 The optimum pH for enzyme activity was determined by measuring the initial rate of hydrolysis of the .sup.32 P-labeled Flap substrate (FIG. 2) by the enzyme at 50.degree. C. in 50 mM sodium acetate buffer, 50 mM phosphate buffer and 50 mM borate buffer (pH 4 to 9), respectively. Because the maximum initial rate was achieved in the vicinity of pH 6.0 to 8.0, it was concluded that the optimum pH was 6.0 to 8.0.

55 (3) Optimum Temperature

56 A predetermined amount of the enzyme was added to 50 mM phosphate buffer, pH 8.0 containing 400 fmol .sup.32 P-labeled Flap substrate (FIG. 2) as the substrate and reacted for 30 minutes to examine its relative activity. The maximum activity (optimum temperature) was 75.degree. C. or more.

57 (4) Thermostability

58 An aqueous solution containing the enzyme at a concentration of 0.1 mg/ml (50 mM phosphate buffer, pH 8.0, 1 M NaCl) was heated at 95.degree. C. for 5 hours and then examined for its residual activity. The result indicated that the half-life of the activity was 5 hours. Further, a differential scanning colorimeter (DSC) was used to examine thermal denaturation. When 0.5 mg/ml of the enzyme solution (50 mM phosphate buffer, pH 8.0, 1 M NaCl) was measured at 0 to 125.degree. C., it was revealed that the thermal denaturation temperature (Tm) is 103.degree. C.

SEQUENCE LISTING

<100> GENERAL INFORMATION:

<160> NUMBER OF SEQ ID NOS: 7

<200> SEQUENCE CHARACTERISTICS:

<210> SEQ ID NO 6

<211> LENGTH: 16

<212> TYPE: DNA

<213> ORGANISM: Artificial Sequence

<220> FEATURE:

<223> OTHER INFORMATION: Description of Artificial Sequencesynthetic oligonucleotide used for the preparation of the Flap substrate

<400> SEQUENCE: 6

cacgttgact accgtc 16

<200> SEQUENCE CHARACTERISTICS:

<210> SEQ ID NO 7

<211> LENGTH: 34

<212> TYPE: DNA

<213> ORGANISM: Artificial Sequence

<220> FEATURE:

<223> OTHER INFORMATION: Description of Artificial Sequencesynthetic oligonucleotide used for the preparation of the Flap substrate

<400> SEQUENCE: 7

gatgtcaagc agtcctaact ttgaggcaga gtcc 34

CLAIMS:

What is claimed is:

1. DNA coding for a protein comprising the amino acid sequence show in SEQ ID NO:2.

2. DNA comprising the nucleotide sequence show in SEQ ID NO:1.

3. A recombinant vector comprising the DNA of claim 1.

4. A transformant transformed with the recombinant vector of claim 3.

5. A process for producing a thermostable Flap endonuclease, which comprises culturing the transformant of claim 4 in a medium to produce the thermostable Flap endonuclease.

6. A recombinant vector comprising the DNA of claim 2.

7. DNA encoding a thermostable Flap endonuclease that is obtainable from a hyperthermophile bacterium belonging to the genus Pyrococcus, the optimum temperature of said endonuclease being 75.degree. C. or more.

8. A method for treating nucleic acid in a high-temperature environment, comprising (1) providing a nucleic acid substrate with one or more cleavage structures and then, at a temperature in the range of at least 75.degree. C., (2) reacting said nucleic acid substrate with a thermostable Flap endonuclease which has an optimum temperature in said range.

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Search Results - Record(s) 11 through 20 of 20 returned.

☐ 11. Document ID: US 20020102591 A1

Using default format because multiple data bases are involved.

L3: Entry 11 of 20 File: PGPB Aug 1, 2002

PGPUB-DOCUMENT-NUMBER: 20020102591
PGPUB-FILING-TYPE: new
DOCUMENT-IDENTIFIER: US 20020102591 A1

TITLE: Methods for detection of a target nucleic acid

PUBLICATION-DATE: August 1, 2002

INVENTOR-INFORMATION:

NAME	CITY	STATE	COUNTRY	RULE-47
Sorge, Joseph A.	Wilson	NY	US	

US-CL-CURRENT: 435/6

Full | Title | Citation | Front | Review | Classification | Date | Reference | Sequences | Attachments | Claims | KWMC | Draw De

☐ 12. Document ID: US 6589743 B2

L3: Entry 12 of 20 File: USPT Jul 8, 2003

US-PAT-NO: 6589743
DOCUMENT-IDENTIFIER: US 6589743 B2

TITLE: Methods for detection of a target nucleic acid using a probe comprising secondary structure

DATE-ISSUED: July 8, 2003

INVENTOR-INFORMATION:

NAME	CITY	STATE	ZIP CODE	COUNTRY
Sorge; Joseph A.	Wilson	NY		

US-CL-CURRENT: 435/6; 435/91.2

Full | Title | Citation | Front | Review | Classification | Date | Reference | Sequences | Attachments | Claims | KWMC | Draw De

☐ 13. Document ID: US 6562575 B1

L3: Entry 13 of 20 File: USPT May 13, 2003

US-PAT-NO: 6562575
DOCUMENT-IDENTIFIER: US 6562575 B1

TITLE: Analyte-specific assays based on formation of a replicase substrate

DATE-ISSUED: May 13, 2003

INVENTOR-INFORMATION:

NAME	CITY	STATE	ZIP CODE	COUNTRY
Dahl; Gary A.	Madison	WI		

US-CL-CURRENT: 435/6; 435/91.1

Full | Title | Citation | Front | Review | Classification | Date | Reference | Sequences | Attachments | Claims | KWIC | Draw De

---

☐ 14. Document ID: US 6548250 B1

L3: Entry 14 of 20 File: USPT Apr 15, 2003

US-PAT-NO: 6548250
DOCUMENT-IDENTIFIER: US 6548250 B1

TITLE: Methods for detection of a target nucleic acid sequence

DATE-ISSUED: April 15, 2003

INVENTOR-INFORMATION:

NAME	CITY	STATE	ZIP CODE	COUNTRY
Sorge; Joseph A.	Wilson	WY		

US-CL-CURRENT: 435/6; 435/18, 435/183, 435/194, 435/195, 435/196, 435/4, 435/810, 435/822, 435/91.53, 436/94, 530/350

Full | Title | Citation | Front | Review | Classification | Date | Reference | Sequences | Attachments | Claims | KWIC | Draw De

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☐ 15. Document ID: US 6528254 B1

L3: Entry 15 of 20 File: USPT Mar 4, 2003

US-PAT-NO: 6528254
DOCUMENT-IDENTIFIER: US 6528254 B1

TITLE: Methods for detection of a target nucleic acid sequence

DATE-ISSUED: March 4, 2003

INVENTOR-INFORMATION:

NAME	CITY	STATE	ZIP CODE	COUNTRY
Sorge; Joseph A.	Wilson	WY		

US-CL-CURRENT: 435/6; 435/183, 435/91.1, 436/94, 536/23.1, 536/24.3, 536/24.33

Full | Title | Citation | Front | Review | Classification | Date | Reference | Claims | KWMC | Draw De

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☐ 16. Document ID: US 6492161 B1

L3: Entry 16 of 20 File: USPT Dec 10, 2002

US-PAT-NO: 6492161
DOCUMENT-IDENTIFIER: US 6492161 B1

TITLE: Bacteriophage RM 378 of a thermophilic host organism

DATE-ISSUED: December 10, 2002

INVENTOR-INFORMATION:

NAME	CITY	STATE	ZIP CODE	COUNTRY
Hjorleifsdottir; Sigridur	Reykjavik			IS
Hreggvidsson; Gudmundur O.	Reykjavik			IS
Fridjonsson; Olafur H.	Reykjavik			IS
Aevarsson; Arnthor	Hveragerdi			IS
Kristjansson; Jakob K.	Reykjavik			IS

US-CL-CURRENT: 435/235.1; 435/320.1, 436/94, 536/23.1

Full | Title | Citation | Front | Review | Classification | Date | Reference | Claims | KWMC | Draw De

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☐ 17. Document ID: US 6350580 B1

L3: Entry 17 of 20 File: USPT Feb 26, 2002

US-PAT-NO: 6350580
DOCUMENT-IDENTIFIER: US 6350580 B1
**** See image for Certificate of Correction ****

TITLE: Methods for detection of a target nucleic acid using a probe comprising secondary structure

DATE-ISSUED: February 26, 2002

INVENTOR-INFORMATION:

NAME	CITY	STATE	ZIP CODE	COUNTRY
Sorge; Joseph A.	Wilson	NY		

US-CL-CURRENT: 435/6; 435/91.1, 435/91.2

Full | Title | Citation | Front | Review | Classification | Date | Reference | Sequences | Attachments | Claims | KWIC | Draw De

☐ 18. Document ID: US 6255081 B1

L3: Entry 18 of 20 File: USPT Jul 3, 2001

US-PAT-NO: 6255081
DOCUMENT-IDENTIFIER: US 6255081 B1

TITLE: Thermostable flap endonuclease derived from hyperthermophile bacterium belonging to the genus pyrococcus

DATE-ISSUED: July 3, 2001

INVENTOR-INFORMATION:

NAME	CITY	STATE	ZIP CODE	COUNTRY
Matsui; Ikuo	Ibaraki			JP
Ishikawa; Kazuhiko	Ibaraki			JP
Kosugi; Yoshitsugu	Ibaraki			JP
Matsui; Eriko	Ibaraki			JP
Kawasaki; Satoko	Chiba			JP

US-CL-CURRENT: 435/91.1; 435/199, 435/252.3, 435/252.33, 435/320.1, 536/23.2

Full | Title | Citation | Front | Review | Classification | Date | Reference | Sequences | Attachments | Claims | KWIC | Draw De

☐ 19. Document ID: US 6251649 B1

L3: Entry 19 of 20 File: USPT Jun 26, 2001

US-PAT-NO: 6251649
DOCUMENT-IDENTIFIER: US 6251649 B1

TITLE: Thermostable flap endonuclease derived from a hyperthermophile bacterium belonging to the genus pyrococcus

DATE-ISSUED: June 26, 2001

INVENTOR-INFORMATION:

NAME	CITY	STATE	ZIP CODE	COUNTRY
Matsui; Ikuo	Ibaraki			JP
Ishikawa; Kazuhiko	Ibaraki			JP
Kosugi; Yoshitsugu	Ibaraki			JP
Matsui; Eriko	Ibaraki			JP
Kawasaki; Satoko	Chiba			JP

US-CL-CURRENT: 435/199

Full | Title | Citation | Front | Review | Classification | Date | Reference | Sequences | Attachments | Claims | KWIC | Draw De

☐ 20. Document ID: US 5874283 A

L3: Entry 20 of 20 File: USPT Feb 23, 1999

US-PAT-NO: 5874283
DOCUMENT-IDENTIFIER: US 5874283 A

TITLE: Mammalian flap-specific endonuclease

DATE-ISSUED: February 23, 1999

INVENTOR-INFORMATION:

NAME	CITY	STATE	ZIP CODE	COUNTRY
Harrington; John Joseph	Shaker Heights	OH	44120	
Hsieh; Chih-Lin	St. Louis	MO	63131	
Lieber; Michael R.	St. Louis	MO	63131	

US-CL-CURRENT: 435/252.3; 435/199, 435/252.33, 435/320.1, 435/69.1, 530/350, 536/23.2, 536/23.5

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Terms	Documents
double with flap with DNA	20

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L3: Entry 15 of 20 File: USPT Mar 4, 2003

US-PAT-NO: 6528254
DOCUMENT-IDENTIFIER: US 6528254 B1

TITLE: Methods for detection of a target nucleic acid sequence

DATE-ISSUED: March 4, 2003

INVENTOR-INFORMATION:

NAME	CITY	STATE	ZIP CODE	COUNTRY
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CLAIMS:

What is claimed is:

1. A method of generating a signal indicative of the presence of a target nucleic acid sequence in a sample, comprising forming a cleavage structure comprising duplex and single-stranded nucleic acid by incubating a sample comprising a target nucleic acid sequence with a nucleic acid polymerase and cleaving said cleavage structure with a FEN nuclease to generate a signal, wherein generation of said signal is indicative of the presence of a target nucleic acid sequence in said sample.

2. The method of claim 1 wherein said nucleic acid polymerase substantially lacks 5' to 3' exonuclease activity.

3. A method of detecting or measuring a target nucleic acid sequence comprising forming a cleavage structure comprising duplex and single-stranded nucleic acid by incubating a sample comprising a target nucleic acid sequence with a nucleic acid polymerase, cleaving said cleavage structure with a FEN nuclease to release a nucleic acid fragment and detecting and/or measuring the release of said fragment as an indication of the presence of the target sequence in the sample.

4. The method of claim 3 wherein said nucleic acid polymerase substantially lacks 5' to 3' exonuclease activity.

5. The method of claim 1 or 3 wherein the nucleic acid polymerase is a DNA polymerase.

6. The method of claim 1 or 3 wherein the nucleic acid polymerase is thermostable.

7. The method of claim 1 or 3 wherein the FEN nuclease is a flap-specific nuclease.

8. The method of claim 1 or 3 wherein the FEN nuclease is thermostable.

9. The method of claim 1 or 3 wherein a cleavage structure is formed comprising at least one labeled moiety capable of providing a signal.

10. The method of claim 1 or 3 wherein a cleavage structure is formed comprising a pair of interactive signal generating labeled moieties effectively positioned to quench the generation of a detectable signal, said labeled moieties being separated by a site susceptible to FEN nuclease cleavage, thereby allowing the nuclease activity of the FEN nuclease to separate the first interactive signal generating labeled moiety from the second interactive signal generating labeled moiety by cleaving at said site susceptible to FEN nuclease, thereby generating a detectable signal.

11. The method of claim 10 wherein said pair of interactive signal generating moieties comprises a quencher moiety and a fluorescent moiety.

12. The method of claim 1 wherein a cleavage structure comprises at least one oligonucleotide primer.

13. A polymerase chain reaction process for detecting a target nucleic acid sequence in a sample comprising providing a cleavage structure comprising duplex and single-stranded nucleic acid, providing a set of oligonucleotide primers wherein a first primer contains a sequence complementary to a region in one strand of the target nucleic acid sequence and primes the synthesis of a complementary DNA strand, and a second primer contains a sequence complementary to a region in a second strand of the target nucleic acid sequence and primes the synthesis of a complementary DNA strand and amplifying the target nucleic acid sequence employing a nucleic acid polymerase as a template-dependent polymerizing agent under conditions which are permissive for PCR cycling steps of (i) annealing of primers required for amplification to a template nucleic acid sequence contained within the target nucleic acid sequence, (ii) extending the primers wherein said nucleic acid polymerase synthesizes a primer extension product, and (iii) cleaving said cleavage structure employing a FEN nuclease as a cleavage agent for release of labeled fragments from said cleavage structure thereby creating detectable labeled fragments and detecting and/or measuring the release of labeled fragments as an indication of the presence of the target sequence in the sample.

14. The polymerase chain reaction process of claim 13 wherein said nucleic acid polymerase substantially lacks 5' to 3' exonuclease activity.

15. The method of claim 13 wherein said oligonucleotide primers of step b are oriented such that the forward primer is located upstream of said cleavage structure and the reverse primer is located downstream of said cleavage structure.

16. The polymerase chain reaction process of claim 13 wherein the nucleic acid polymerase is a DNA polymerase.

17. The polymerase chain reaction process of claim 13 wherein the nucleic acid polymerase is thermostable.

18. The polymerase chain reaction process of claim 13 wherein the nucleic acid polymerase is selected from the group consisting of 5' to 3' exonuclease deficient Taq polymerase and Pfu polymerase.

19. The polymerase chain reaction process of claim 13 wherein the FEN nuclease is thermostable.

20. The polymerase chain reaction process of claim 13 wherein the FEN nuclease is a flap-specific nuclease.

21. The polymerase chain reaction process of claim 13 wherein the FEN nuclease is selected from the group consisting of FEN nuclease enzyme derived from Archaeglobus fulgidus, Methanococcus jannaschii, Pyrococcus furiosus, Taq, Tfl and Bca.

22. The polymerase chain reaction process of claim 13 wherein the labeled cleavage structure is formed by the addition of at least one labeled moiety capable of providing a signal.

23. A polymerase chain reaction process for simultaneously forming a cleavage structure comprising duplex and single-stranded nucleic acid, amplifying a target nucleic acid sequence in a sample and cleaving said cleavage structure comprising: (a) providing an upstream oligonucleotide primer complementary to a region in one strand of the target nucleic acid sequence and a downstream labeled probe complementary to a region in the same strand of the target nucleic acid sequence, wherein the upstream primer contains a sequence complementary to a region in one strand of the target nucleic acid sequence and primes the synthesis of a complementary DNA strand, and the downstream probe contains a sequence complementary to a region in a second strand of the target nucleic acid sequence and primes the synthesis of a complementary DNA strand; and (b) detecting a nucleic acid which is produced in a reaction comprising amplification of said target nucleic acid sequence and cleavage thereof wherein a nucleic acid polymerase is a template-dependent polymerizing agent under conditions which are permissive for PCR cycling steps of (i) annealing of primers to a target nucleic acid sequence, (ii) extending the primers of step (a) wherein said nucleic acid polymerase synthesizes primer extension products, and wherein the primer extension product of the primer of step (a) partially displaces the downstream probe of step (a) to form a cleavage structure comprising duplex and single-stranded nucleic acid; and (iii) cleaving said cleavage structure employing a FEN nuclease as a cleavage agent for release of labeled fragments from said cleavage structure thereby creating detectable labeled fragments.

24. The polymerase chain reaction process of claim 23 wherein said nucleic acid polymerase substantially lacks 5' to 3' exonuclease activity.

25. A method of forming a cleavage structure comprising providing a target nucleic acid sequence, providing an upstream primer complementary to said target nucleic acid sequence, providing a downstream probe complementary to said target nucleic acid sequence, extending the 3' end of the upstream primer with a nucleic acid polymerase substantially lacking 5' to 3' exonuclease activity; and displacing the 5' end of the downstream probe.

26. A kit for generating a signal indicative of the presence of a target nucleic acid sequence in a sample, comprising a FEN nuclease, a suitable buffer and a nucleic acid polymerase that substantially lacks 5' to 3' exonuclease activity, wherein said FEN nuclease, said buffer and said nucleic acid polymerase are in the same composition.

27. The kit of claim 26 wherein said nucleic acid polymerase is thermostable.

28. The kit of claim 26 wherein said FEN nuclease is thermostable.

29. The kit of claim 26 further comprising a labeled nucleic acid complementary to said target nucleic acid sequence.

30. A composition comprising a nucleic acid polymerase, a FEN nuclease, and a nucleic acid primer.

31. The composition of claim 30 wherein said nucleic acid polymerase substantially lacks 5' to 3' exonuclease activity.

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